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(54) Title: SELECTIVE ALTERATION OF ANTIBODY IMMUNOGENICITY

(57) Abstract

The present invention relates to a simple process for the modification of, e.g., anti-TAA antibodies, which alters their immunogenicity so that their ability to induce an anti-isotypic response is selectively diminished, while they remain able to elecit an anti-idiotypic response. The latter is of potential immunotherapeutic value, i.e., by activation of the idiotype-anti-idiotype network. This modification takes the form of a controlled and partial reduction of the antibody; effector regions are retained. The invention should permit repeat injections (for diagnosis and therapy) and reduce HAMA interference in serodiagnostic assays.

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SELECTIVE ALTERATION OF ANTIBODY IMMUNOGENICITY

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to a method of altering the immunogenicity of antibodies so that, upon administration to a suitable subject, an immune response is elicited which is predominantly anti-idiotypic rather than anti-isotypic in character.

Description of the Background Art

All vertebrates possess a surveillance mechanism, called the immune system, that protects them from pathogenic microorganisms (including viruses), multicellular parasites, and cancer cells. The immune system specifically recognizes and selectively eliminates these undesirables by a process known as the immune response. One of its two important subsystems is the humoral immune system, which relies on antibodies, produced in quantity by plasma cells, that circulate through the blood and the lymphatic fluid.

The first step in the immune response is the recognition of the presence of a foreign entity. Antigens are molecules which are subject to immune recognition. The portion of an antigen to which an antibody binds is called its antigenic determinant, or epitope. Not all antigens are capable of eliciting a response, as opposed to simple molecular recognition, from the immune system. Antigens which can elicit an immune response are termed immunogens, and are usually macromolecules, such as proteins, nucleic acids, carbohydrates, and lipids, of at least 5000 Daltons molecular weight. However, many small nonimmunogenic molecules, termed haptens, can stimulate an immune response if associated with a large carrier molecule.

Antibodies, also known as immunoglobulins, are proteins. They have two principal functions. The first is to recognize (bind) foreign antigens. The second is to mobilize other elements of the immune system to destroy the foreign entity.

The basic unit of immunoglobulin structure is a complex of four polypeptides -- two identical low molecular weight ("light") chains and two identical high molecular weight ("heavy") chains,

linked together by both nocovalent associations and by disulfide bonds. Different antibodies will have anywhere from one to five of these basic units. The immunoglobulin unit may be represented schematically as a "Y". Each branch of the "Y" is formed by the amino terminal portion of a heavy chain and an associated light chain. The base of the "Y" is formed by the carboxy terminal portions of the two heavy chains. The node of the "Y" is the so-called hinge region, and is quite flexible. Five human antibody classes (IgG, IgA, IgM, IgD and IgE), and within these classes, various subclasses, are recognized on the basis of structural differences, such as the number of immunoglobulin units in a single antibody molecule, the disulfide bridge structure of the individual units, and differences in chain length and sequence. The class and subclass of an antibody is its isotype.

The amino terminal regions of the heavy and light chains are far more diverse in sequence than the carboxy terminal regions, and hence are termed the variable domains. This is the part of the antibody whose structure confers the antigen-binding specificity of the antibody. A heavy variable domain and a light variable domain together form a single antigen-binding site, thus, the basic immunoglobulin unit has two antigen-binding sites. The walls of the antigen-binding site are defined by hypervariable segments of the heavy and light variable domains. Binding site diversity is generated both by sequence variation in the hypervariable region and by random combinatorial association of a heavy chain with a light chain. Collectively, the hypervariable segments are termed the paratope of the antibody; this paratope is essentially complementary to the epitope of the cognate antigen.

The carboxy terminal portion of the heavy and light chains form the constant domains. While there is much less diversity in these domains, there are, first of all, differences from one animal species to another, and secondly, within the same individual, there will be several different isotypes of antibody, each having a different function.

The IgG molecule may be divided into homology units. The light chain has two such units, the V_L and C_L, , and the heavy chain has four, designated V_H, C_H1, C_H2 and C_H3. All are about

110 amino acids in length and have a centrally located intrachain disulfide bridge that spans about 60 amino acid residues. The sequences of the two V-region homology units are similar, as are the sequences of the four C-region homology units. These homology units in turn form domains. The two variable domains have already been mentioned; there are also four constant domains. Mild proteolytic digestion of IgG results in the production of certain fragments of interest. V-C1 is Fab; C_H2-C_H3 is Fc; $(V\text{-}C1)_2$ is $(Fab')_2$, V-C1-C2 is Fabc, and V alone is Fv.

While the variable domains are responsible for antigen binding, the constant domains are charged with the various effector functions: stimulation of B cells to undergo proliferation and differentiation, activation of the complement cell lysis system, opsonization, attraction of macrophages to ingest the invader, etc. Antibodies of different isotypes have different constant domains and therefore have different effector functions. The best studied isotypes are IgG and IgM.

If a specific antibody from one animal is injected as an immunogen into a suitable second animal, the injected antibody will elicit an immune response. Some of these anti-antibodies will be specific for the unique epitopes (idiotopes) of the variable domains of the injected antibodies; these epitopes are known collectively as the idiotype of the primary antibody and the secondary (anti-) antibodies which bind to these epitopes are known as anti-idiotypic antibodies. Other secondary antibodies will be specific for the epitopes of the constant domains of the injected antibodies and hence are known as anti-isotypic antibodies. (The term "anti-isotypic" antibodies, as used herein, includes antibodies that are merely species-specific as well as antibodies which are also class or subclass-specific.)

The "network" theory states that antibodies produced initially during an immune response will carry unique new epitopes to which the organism is not tolerant, and therefore will elicit production of secondary antibodies (Ab2) directed against the idiotypes of the primary antibodies (Ab1). These secondary antibodies likewise will have an idiotype, which will induce production of tertiary antibodies (Ab3), and so forth.

It also suggests that some of those secondary antibodies will have a binding site which is the complement of the complement of the original antigen, and thus will reproduce the "internal image" of the original antigen. In other words, an anti-idiotypic antibody may be a surrogate antigen.

There are four major types of anti-idiotypic antibodies. The alpha-type is one which binds an epitope remote from the paratope of the primary antibody. The beta-type is one whose paratope mimicks the epitope of the original antigen. The gamma-type binds near enough to the paratope of the primary antibody to interfere with antigen binding. The epsilon type recognizes an idiotypic determinant that mimicks a constant domain antigenic structure. Moreover, anti-isotypic antibodies may be heavy chain-specific or light chain-specific.

"Active immunotherapy" is the administration of an antigen, in the form of a vaccine, to a patient, so as to elicit a protective immune response. "Passive immunotherapy" involves the administration of antibodies to a patient. Antibody therapy is conventionally characterized as passive since the patient is not the source of the antibodies. However, the term passive is misleading because the patient can produce anti-idiotypic secondary antibodies which in turn provoke an immune response which is cross-reactive with the original antigen.

As stated by Koprowski (3), a traditional approach to cancer immunotherapy is to administer anti-tumor antibodies, i.e., antibodies which recognize an epitope on a tumor cell, to patients. However, the development of the "network" theory led her and others (4) to suggest the direct administration of exogenously produced anti-idiotype antibodies, that is antibodies raised against the idiotype of an anti-tumor antibody. Koprowski assumes that the patient's body will produce anti-antibodies which will not only recognize these anti-idiotype antibodies, but also the original tumor epitope.

Koprowski's exogenous anti-idiotypic antibodies are the product of a rather complex production process. Polyclonal anti-idiotypic antibodies must be separated from other antibodies in the serum of the animal. The use of monoclonal anti-idiotypic antibodies simplifies purification to some degree, but at the

cost of a laborious screening procedure to identify hybridomas secreting the desired anti-idiotypic antibody. Then these cells must be expanded in culture. Finally, once a production culture is developed, the antibodies still must be recovered, purified and tested. Applicants believe it to be preferable to stimulate in vivo production of the anti-idiotypic antibody.

It is of course true that Applicants' antibodies must also be purified. However, Applicants need only distinguish between antibodies which bind to the immunogen and those which do not. The proponents of exogenous anti-idiotypic antibody therapy must differentiate antibodies which bind to the same immunogen, but in different places.

In a related vein, it has been suggested that one may administer a synthetic polypeptide that substantially immunologically corresponds to an idiotypic epitope of an antibody directed against an antigen of interest (5). However, this polypeptide must be synthesized and purified. Moreover, this methodology requires knowledge of the sequence of the antigen binding site of the anti-idiotypic antibody.

Sources of human antibodies are limited to subjects already suffering from the disease of interest, as it is unethical to introduce a disease into a subject merely so the subject will begin producing antibodies which may be harvested. Because of the difficulties of collecting human antibodies, clinicians rely on antibodies of nonhuman origin, such as mouse antibodies. Unfortunately, besides eliciting an anti-idiotypic response, these mouse antibodies, when administered to humans, also provoke production of secondary human anti-mouse antibodies (HAMA) directed against mouse-specific and mouse isotype-specific portions of the primary antibody molecule. This immune reaction occurs because of differences in the primary amino acid sequences in the constant regions of the immunoglobulins of mice and humans. Both IgG and IgM subclasses of HAMA have been detected. The IgG response appears later, is longer-lived than the typical IgM response, and is more resistant to removal by plasmapheresis.

Clinically, the development of HAMA increases the likelihood of anaphylactic or serum sickness-like reactions to subsequent

administration of murine immunoglobulins. These secondary antibodies reduce the efficacy of repeat immunotherapy by complexing subsequently administered mouse antibody (31). HAMA-induced increases in the clearance of the injected antibody or fragment can result in reduced tumor localization, enhanced uptake into liver and spleen, and tumor escape from therapy. HAMA can also cause interference with immunodiagnosis, and thereby hinder monitoring of the progress of the disease and the effectiveness of the course of treatment.

The anti-isotype response has been avoided in prior immunoimaging work through the use of monovalent Fab fragments or divalent $(Fab')_2$ fragments. These fragments lack most of the constant region and therefore present only a very limited opportunity for anti-isotype binding (1). Moreover, they lack the effector functions of a more intact antibody and therefore will not activate complement, or bind to an Fc receptor on a killer cell. Accordingly, such fragments, which lack most or all of the constant region, are not normally used in immunotherapy.

Another approach is to conjugate a tolerogen, such as polyethylene glycol, to the antibody to reduce its immunogenicity (2). Unfortunately, PEGging an antibody also diminishes its ability to elicit an anti-idiotypic response.

Wagner, et al. (6) radioimmunoimaged 12 patients with ovarian carcinomas using Iodine-131 labeled $F(ab')_2$ fragments of the anti-CA125 mouse antibody OC125. All patients had been treated in the same manner by surgery followed by chemotherapy. Five of the patients developed anti-idiotypic antibodies against the imaging antibody. In 1989, only these five patients were still alive. Wagner, et al. suggested that their longterm survival was attributable to their development of anti-idiotypic antibodies against the OC125 fragments, and hence to induction of the idiotypic network. While Wagner et al.'s fragments may have exerted a serendipitous immunotherapeutic effect through generation of Ab3, they nonetheless lack the effector functions of conventional immunotherapeutic agents. Moreover, because these fragments are more rapidly cleared from the bloodstream, they are less useful than intact antibody for immunotherapy.

The use of intact antibody (Ab1) to activate the idiotype-anti-idiotype network, while potentially enhancing the immunotherapeutic utility of the antibody, would raise the issue of problems with anti-isotypic responses, as previously mentioned. Wagner et al. did not need to address the possibility of an anti-isotypic response since he had administered fragments lacking most of the constant region.

A methodology is urgently needed that allows use of animal antibodies in human therapy, with in vivo stimulation of an endogenous anti-idiotypic response and without concomitant stimulation of a substantial anti-isotypic response (the term here including a species-specific response), which does not require use of antibody fragments which lack constant regions.

All references, including patents and patent applications, which are cited anywhere in this specification are hereby incorporated by reference. No admission is made that any cited reference constitutes prior art, or pertinent prior art.

SUMMARY OF THE INVENTION

Applicants have discovered that the immunogenic character of antibodies may be modified so as to substantially eliminate the anti-isotype response while substantially preserving the anti-idiotype response to the antibodies.

If the anti-isotype response is eliminated, it may be possible to repeatedly administer an antibody to a patient without fear of putting the patient into anaphylactic shock brought on by an adverse immune reaction between the exogenous antibody and previously elicited anti-isotype anti-antibodies. Retention of the anti-idiotype response is advantageous, however, as the anti-idiotype anti-antibody mimics the original antigen, and thereby can elicit production in the patient of endogenous antibodies which likewise recognize the original antigen. Elimination of the anti-isotypic response will also facilitate subsequent immunosurveillance of the patient by <u>in vitro</u> and <u>in vivo</u> immunodiagnostic techniques, as interference from anti-isotypic anti-antibodies will be avoided.

While simply removing the Fc portion of an antibody is likely to substantially eliminate its ability to elicit an anti-isotype response, the use of antibody fragments such as Fab and Fab' fragments has other disadvantages. These fragments have a shorter residency time in the bloodstream, and therefore are less desirable from a therapeutic standpoint than a whole antibody. They also fail to provide all of the effector functions associated with intact antibody, which reduces their therapeutic effectiveness. Indeed, they may actually interfere with the action of endogenous antibodies, which have the effector function, by blocking the antigenic determinants. Thus, while they have some therapeutic value through eliciting production of Ab3, in general they are not suitable as immunotherapeutic agents.

Instead, applicants treat the antibody with a reagent that is capable of reducing certain of the disulfide (-S-S-) bridges of the immunoglobulin, thereby generating free sulfhydryl groups, but without fragmenting the antibody sufficiently to abolish effector function.

The reduction also results in a denaturation of the heavy chain conformation, and thereby substantially eliminates anti-heavy chain or isotype antibody response. It is also believed that under certain circumstances the anti-idiotypic response can be increased in both an absolute as well as a relative sense. While applicants do not wish to be bound to this theory, it is believed that the cleavage of certain disulfides results in greater conformational flexibility in the critical antigen binding variable and hypervariable regions, exposing areas which previously were subject to steric hindrance, and therefore to a greater propensity toward anti-idiotype responses. However, an absolute increase in the anti-idiotypic response is not required for the practice of this invention.

The present invention also relates to an improved method of reducing, and, if desired, radiolabeling antibodies. These antibodies may be used for radioimmunotherapy, or for radioimmunoimaging (with a reduced isotypic HAMA response to interfere with subsequent immunotherapy).

The appended claims are hereby incorporated by reference as a further recitation of the preferred embodiments.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to the production of reduced antibodies and their use, alone or in combination with other agents, as immunotherapeutic agents.

All immunoglobulin G molecules consist of two heavy and two light polypeptide chains covalently bound to each other through several disulphide bridges between cysteine amino acids. In addition to these interchain bridges, there are a greater number of intrachain disulphide bonds which also aid in the maintenance of the tertiary structure of the molecule. Under reductive conditions, these bridges can be cleaved to the corresponding sulphydryl forms.

There are numerous techniques for preparing reduced antibodies. In general, the compounds used fall into three categories - the classical reducing agents comprising organic (for example, formamidine sulfonic acid) and inorganic (for example, mercurous ion, stannous ion, cyanide ion, sodium

cyanoborohydride, sodium borohydride, etc.) compounds, the thiol exchange reagents (for example, dithiothreitol, mercaptoethanol, mercaptoethanolamine) and protein reductants (for example, thioredoxin). Exposure of immunoglobulin-G molecules (or their fragments) to these compounds results in somewhat selective reduction of disulphides to form various sulphydryl groups. Under continuing reductive conditions, these sulphydryl groups remain, resulting in an at least partially disulphide reduced protein molecule, and at least potentially changing the tertiary structure of the immunoglobulin. The effect of the reduction on the conformation and immunoreactivity of the antibody molecule is dependent on the degree of reduction.

The reduction results in a denaturation of the heavy chain conformation, and thereby substantially reduces or even eliminates anti-heavy chain or isotype antibody response.

While totally reduced antibody molecules are potentially usable, it is likely that their affinity for antigen will be substantially diminished. Consequently, it is preferable to control the degree of reduction of the antibody so that it retains at least some of its intra- and/or inter-chain disulphide bonds. The most susceptible disulphide bridges are those in the hinge region and therefore under appropriate conditions these can be preferentially cleaved. This potentially allows greater movement of the critical antigen binding variable and hypervariable regions and may expose previously hindered areas of these regions. With some antibodies, this may lead to an enhancement of the anti-idiotype human anti-mouse antibody response.

Reducing agents potentially useful for the selective elimination of the isotype immunogenicity of the antibody are readily tested for suitability by the HAMA assay described in this specification, or by other assays capable of differentiating anti-idiotypic and anti-isotypic HAMA (31).

The HAMA assay described in the Examples is a two-step indeirect radioimmunoassay. Beads which have been precoated with goat anti-mouse antibody are incubated with a second murine antibody or fragment to form the complex that captures HAMA. In order to measure a generalized HAMA response, only a nonspecific

antibody, e.g. an irrelevant murine IgG monoclonal antibody, is used as the second antibody. In order to measure an anti-idiotypic HAMA response, the particular antibody administered to the patients is used on some beads and the nonspecific control antibody is used on others.

After the incubation with the second murine antibody or fragment, the beads are washed to remove any unbound antibodies. The beads are now considered "primed" to capture HAMA. After washing, diluted test serum is added and incubated with the primed beads. HAMA present in the serum is captured or linked to the primed beads during this incubation. Following a second wash, the beads are incubated with a radiolabeled tracer antibody, e.g., Iodine-125 labeled polyclonal anti-human antibodies, which binds to captured HAMA. Any unbound radiolabeled antibody is removed by a final wash before measuring the amount of bound radioactivity.

Results obtained using the positive (anti-mouse Ig serum) and negative (human serum) controls supplied in the kit are used to calculate the HAMA limit.

About 9% of a normal population has been found to exhibit positive HAMA responses before in vivo administration of murine immunoglobulin. Certain patient groups have higher preinjection HAMA responses, so it is desirable to obtain a pre-injection baseline sample.

The present invention is not limited to any particular method of determining anti-isotypic and anti-idiotypic HAMA, or any particular reagents for use therein. It is believed that the Behringerwerke ENZYGNOST HAMA micro assay has the components needful for measuring both HAMA responses, though the kit does not explain how to perform this calculation. Measurement of anti-idiotypic response is reported in, e.g., Reinsberg, et al., Clin. Chem. ,36: 164-167 (1990); GOldman-Leikin, et al., Exp. Hematol. 16: 861-864 (1988).

While we have spoken in terms of the HAMA response, we could as well have addressed any immune response of one animal to antibodies derived from a different species of animal.

The reduced antibody elicits at least some anti-idiotypic anti-antibody response but no more than a substantially

decreased, if any, anti-isotype response, relative to the unreduced antibody. Desirably, no more than 20%, and more desirably, no more than 5%, of the anti-isotypic response of the subject to the antibody is retained after reduction. Most desirably, the anti-isotypic response is essentially eliminated. Preferably, at least 25%, more preferably at least 50%, still more preferably at least 80%, and most preferably, at least 95%, of the anti-idiotypic response of the subject to the antibody is left under these circumstances. Preferably, the reduction in the anti-isotypic response is substantially greater than the reduction in the anti-idiotypic response.

While it is preferable that the reduced antibodies of the present invention retain their Fc and hinge regions, it is also possible to reduce antibody fragments that possess only a portion of the normal Fc region or hinge region, such as $(Fab')_2$.

If desired, the reduced antibody may be radiolabeled with pertechnetate or perrhenate to produce a radiolabeled antibody which may be used for radioimmunoimaging as well as radioimmunotherapy. The radioisotope may be one with a cytotoxic effect and therefore of therapeutic value if the antibody is directed against an antigen of an undesirable cell, such as a cancer cell.

A particularly preferred reduction method employs $SnCl_2$ as the reducing agent. Preferably, the molar ratio of this reducing agent to the antibody is in the range of 20:1 to 100:1; the most preferred value is about 40:1. Use of a high level of stannous ion increases the chance of damaging or fragmenting the antibody and also increases the likelihood of Tc-99m-Sn(II) formation competing significantly with the MAb-Tc-99m reaction. The concentration of the antibody may be in the range of 1 to 10 mg/mL; preferably 5mg/mL.

The reaction buffer preferably is a tartrate (e.g., NaK tartrate) buffer; the preferred tartrate concentration is greater than 0.05 and less than about 0.2M; the most desirable value being about 0.1M. The use of phthalate, as suggested by Rhodes, U.S. 4,424,200 and 5,078,985, is unnecessary. The high tartrate concentration stabilizes the Sn(II) ions and retards the oxidation to the Sn(IV) state. As a result, precipitation of

Sn(II) or colloidal formation during buffer preparation is not usually observed. The pH of the buffer may be 4-8; a pH which results in excessive precipitation or cloudiness of the buffer, or which results in degradation and loss of immunoreactivity on the part of the antibody, should be avoided. One of the advantages of the present system is, however, the broad pH range it accommodates, allowing selection of a pH to which the antibody is insensitive. Degassing of the buffer is not essential. The pretreatment buffer is compatible with MAb stored in either normal saline or phosphate-buffered saline (PBS), and therefore the researcher may select whichever storage buffer provides better stability for the MAb.

The incubation is preferably from 8-24 hours and the incubation temperature is preferably in the range of 18-40 deg. C., and most desirably is 37 deg. C.

After this treatment, the reduced antibody may be frozen or lyophilized for storage purposes. When desired, the reduced antibody preparation may be reacted with a pertechnetate salt, e.g., Na salt, for labeling purposes. Radiolabeling efficiencies of over 90% are routinely observed, and the immunoreactivity of the antibody is essentially unaffected.

The antibody may also be incorporated into a conjugate having desirable properties. An example of such a conjugate is an immunotoxin, wherein one moiety is an antibody and another is a toxin. The antibody may target, e.g., a virus-infected cell, and the toxin then kills the cell. Useful toxins include, e.g., ricin and abrin.

The antibody may be directed against any antigen of clinical significance, but preferably is directed against a tumor-, pathogen- or parasite-associated antigen. In the case of a tumor-associated antigen (TAA), the cancer may be of the lung, colon, rectum, breast, ovary, prostate gland, head, neck, bone, immune system, or any other anatomical location. The subject may be a human or animal subject. The antibody may be a polyclonal antibody or a monoclonal antibody. When the subject is a human subject, the antibody may be obtained by immunizing any animal capable of mounting a usable immune response to the antigen. The animal may be a mouse, rat, goat, sheep, rabbit or

other suitable experimental animal. The antigen may be presented in the form of a naturally occurring immunogen, or a synthetic immunogenic conjugate of a hapten and an immunogenic carrier. In the case of a monoclonal antibody, antibody producing cells of the immunized animal may be fused with "immortal" or "immortalized" human or animal cells to obtain a hybridoma which produces the antibody. If desired, the genes encoding one or more of the immunoglobulin chains may be cloned so that the antibody may be produced in different host cells, and if desired, the genes may be mutated so as to alter the sequence and hence the immunological characteristics of the antibody produced.

The antibody may be administered to the patient by any immunologically suitable route, such as intravenous, intraperitoneal, subcutaneous, intramuscular or intralymphatic routes, however the intravenous route is preferred. The clinician may compare the anti-idiotypic and anti-isotypic responses associated with these different routes in determining the most effective route of administration.

Example I

Reduction of Antibody

Stannous ion is a known sulphydryl reductant. We use a stabilized stannous ion solution prepared from stannous chloride and tartrate salt. Controlled reduction with stannous ion of a monoclonal antibody produced a modified MAb preparation containing an average of approximately one sulphydryl group per molecule. Further evidence of sulphydryl creation is the ability of the molecule to radiolabel with Tc-99m in the presence of Tc-99m[(III),(IV)m(V)] complexes, known to form stable bonds with thiol groups. This mild controlled process does not lead to any significant loss of antigen binding properties of the MAb.

A solution containing 2.822 g of Sodium Potassium Tartrate is prepared in 98 ml of sterile water for injection and degassed of dissolved oxygen by bubbling nitrogen gas (5-10 psi) through the solution for 30 minutes. A second solution is prepared containing 1.13 g of stannous chloride in 10.0 ml of 1.0 N HCl. A quantity of 400 μl of this solution is added to the tartrate buffer solution and the mixture adjusted to pH=5.6±0.05 as

measured by a calibrated pH meter by slow addition of 1.0 N NaOH. A quantity of 40 ml of this tartrate stabilized stannous ion solution is added to 60 ml of a 5.0 mg/ml solution of MAb-170 or MAb-B43 (contained in a pH 7.4 NaH_2PO_4 buffered matrix).

MAb-170 (more accurately, MAb170H.82) is a murine monoclonal antibody of the IgG1 kappa isotype that was produced by immunizing BALB/c mice with a synthetic glycoconjugate consisting of a Thomsen-Friedenreich (TF) beta (Galbeta1->3GalNAc) disaccharide hapten coupled to an immunologically suitable carrier (serum albumin). It was selected based on its reactivity with human adenocarcinoma tissue _in vitro_. It clearly reacts with adenocarcinomata of the breast, ovary, endometrium, colon, prostate and some bladder. It also reacts with adenosquamous, small cell and squamous cell lung carcinoma tissue. It is described in more detail in copending Ser. No. 07/153,162, filed May 12, 1988, incorporated by reference herein, which is a continuation of Ser. No. 06/927,277, filed Oct. 27, 1986. MAb-170 has been formulated into a Tc-99m radiolabeled antibody kit (TRUSCINT AD, Biomira, Inc., Edmonton, Alberta, Canada) for radioimmunodiagnosis of adenocarcinomas. See McEwan, et al., Nuclear Medicine Communications, 13: 11-19 (1992). A hybridoma (170H82. R1808) secreting MAb 170 was deposited on July 16, 1991 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 USA, an International Depository Authority under the Budapest Treaty, and assigned the accession number HB 10825. This deposit should not be construed as a license to make, use or sell the hybridoma or MAb 170.

MAb-B43 (more accurately, B43.13) is a murine monoclonal antibody of the IgG1 kappa isotype that was produced by immunizing mice with the CA125 antigen. It was selected for its reactivity to CA 125, an ovarian carcinoma-associated antigen. It inhibits the binding of MAb OC125 to CA125. MAb B43 is reactive with CA125 antigen in biopsy tissue and in serous and endometroid carcinomas of the ovary. It has been formulated into a Tc99m-radiolabeled antibody kit (TRUSCINT OV, Biomira, Inc. Edmonton, Alberta, Canada) for radioimmunodiagnosis of ovarian carcinomas. See Capstick, et al., Int. J. Biol. Markers, 6: 129-135 (1991).

Reference to these two antibodies should not be construed as a limitation on the generality of the present invention.

The headspace of the reaction vessel containing this combination is purged with nitrogen gas and allowed to incubate for about 24 hours. Then, 0.67 ml aliquots of the solution are filtered into 5 ml nitrogen purged sterile vials and frozen at -20°C. Each vial contains nominally 2.0 mg of treated MAb-170 or MAb-B43. The final preparation is sterile, pyrogen-free and suitable for human injection.

Example II

Human Anti-Mouse Antibody (HAMA) Assays

The Biomira TRUQUANT HAMA-RIA kit (Biomira, Inc., Edomonton, ALberta, Canda) is an *in vitro* test for the detection of anti-idiotypic and anti-isotypic human anti-mouse antibodies (HAMA) of either the IgG or IgM subclasses, in human serum. However, the principles of the kit are more broadly applicable to the detection of anti-idiotypic and anti-isotypic antibodies.

The Biomira kit utilizes goat anti-mouse capture reagent on 1/4" polystyrene beads. Of course, other anti-mouse capture reagents could be subtituted for the goat anti-mouse antibody. This allows for capture of (a) idiotype and isotype matched or (b) idiotype mismatched, isotype matched control MAbs. Patient samples are then tested against beads that have been primed with matched and mismatched mouse antibodies. By subtracting the anti-isotype (control) response from the anti-idiotype (or matched) response, the two types of HAMA responses can be determined. Formulae for the calculation of the Total, Control, and Idiotype HAMA Indexes appear below:

Total HAMA Index (calculated using the specific or matched antibody)=CPM Sample on idiotype-specific Ab / HAMA Limit*

Control HAMA Index (calculated using the mismatched antibody)=CPM Sample on idiotype mismatched, isotype-matched Ab / HAMA Limit*

Idiotype Index = Total HAMA Index (specific) - Control HAMA Index (mismatched)

*The HAMA Limit [(0.2 x CPM of the Positive Control) + CPM of the Negative Control] used in the HAMA kit was determined to be the upper limit of normal distribution of samples from patients not injected with mouse antibodies. This run specific cutoff value establishes a level above which a >95% confidence can be used to determine that the result obtained is a true anti-mouse antibody response. The evaluation of the MAb-170 patients was based on a change of the HAMA Index from pre-injection to post injection samples. A significant change is a difference greater than 1 HAMA Index value.

Example III

Anti-Idiotype Serum Assays

The present example shows a reduced antibody elicited almost no anti-isotype response relative to an unreduced antibody. While the reduced antibody also exhibited some reduction of the anti-idiotype response, possibly as a result of cleavage of disulfide bridges near the antigen-binding site, this latter response was still substantial. MAb-170, as described above, was labeled with either Tc-99m or In-111. Labeling with Tc-99m was accomplished by first reducing the antibody as described in Example I and then reacting it with sodium pertechnetate as previously described. Labeling with In-111, to act as a control for the reduced MAb 170, did not involve any reductive process. Instead, MAb 170 was reacted with DTPA anhydride to produce a chelate attachment site for In-111 labeling. The HAMA response to a single 4-8 mg dose was determined.

The results are shown in Table 1 below.

While the HAMA kit used to measure the HAMA response used bead-bound MAb 170 in unreduced form as the capture reagent for anti-idiotype antibodies, substitution of bead-bound reduced MAb 170 did not lead to a significant change in the results obtained.

The HAMA response may also be quantified in terms of the

number of patients seroconverting to production of anti-idiotype or anti-isotype following injection of the antibody. The results are shown in Table 2 below.

Example IV

Correlation of HAMA Idiotype with Cancer Survival

In Table III, ten ovarian cancer patients injected with MAbs (fragment MAb OC 125 and reduced but unfragmented MAb B43) had a mean survival time as of the date of compilation of about three years. Of the ten patients, nine were still alive. Of these nine, two have progressing disease and 7 are stable or free of the disease. This is beyond normal expectations for these patients and is attributed to the presence of anti-idotype MAbs against the injected MAbs.

OC-125 is a murine antibody generated by the immunization of BALB/c mice with a human serous papillary cystadenocarcinoma. OC125 reacts with the CA125 antigen, which has been identified as a high molecular weight glycoprotein found on the cell surface of many ovarian cancers.

For molecular biology and immunology procedures not described above, see Sambrook, et al., Molecular Cloning: A Laboratory Manual (2nd ed., Cold Spring Harbor: 1989); Harlow and Lane, Antibodies: A Laboratory Manual (Cold Spring Harbor: 1988); Ausubel, et al., Current Protocols in Molecular Biology (Wiley Interscience: 1987, 1991).

Example V

Hama Analysis Post MAb 170 and MAb 174 Immunoscintigraphy

In support of previous findings the nonspecific and anti-isotype HAMA seroconversion rates after a single immunoscintigraphy with the reduced antibodies of the present invention is significantly lower than historical results with other antibodies/conjugates. Using the TRUQUANT HAMA RIA to measure the response to a single 1 mg dose, and comparing pre-infusion to post infusion samples, 0/22 patients developed a generalized or non-specific HAMA. Amongst patients infused with partially reduced MAb 170 (n=16), no patients showed anti-isotype or generalized HAMA responses and 2/16 seroconverted in an

idiotype specific manner. Amongst patients infused with partially reduced MAb 174 (n=6) no patients showed generalized HAMA while 1/6 did seroconvert in an idiotype specific manner. While the idiotypic-specific HAMA was less pronounced than for Example I, this may well be attributable to the lower dosage employed. In any event, the isotypic HAMA response was eliminated, while at least some idiotypic HAMA response was retained.

Table 1: **HAMA response after 1 injection of either Tc-99m MAb-170 or In-111 MAb-170**

Injected MAb[C] Group (n=12)	1 Month Post Inj.[A] Isotype[B]	Idiotype[B]	Idiotype	Peak Response[A] Isotype[B]	Idiotype[B]	%Idiotype
MAb-170[D] (Tc-99m labeled; reduced)	-0.07	1.11	106.7	0.1	1.61	94.2
MAb-170 (In-111 labeled; non-reduced control)	0.36	1.79	83.3	1.86	2.19	54.1

A Data was collected from pre-injection samples, 1 month post injection and 2 - 8 months post injection. Data is shown for 1 month post injection and the corresponding sample that showed the peak HAMA response (selected from the 2 - 8 month post injection samples). Mean data was generated from 12 patients in each group.

B Data is expressed in Index Units and has had the pre-injection sample data subtracted prior to reporting.

C The antibody injected was either Tc-99m MAb-170 or In-111 MAb-170 (reduced versus non-reduced antibody).

D A similar anti-isotypic response was obtained with a second control, reduced MAb B43.

Table 2: Number of Patients Seroconverting from pre-injection to post-injection (measured at peak response)

Injected Mab	# Producing Anti-isotype	# Producing Anti-idiotype
Tc-99m MAb-170 (reduced)	0/12	6/12
In-111 MAb-170 (control; non-reduced)	3/12	8/12

Note: The total number of subjects producing HAMA in the Tc-99m MAb group was 6. The total number of subjects producing HAMA in the In-111 MAb group was 8. Of these 8, 3 also produced an anti-isotype response. The point to note is that this response is typical for a first injection of MAb whereas the absence of an anti-isotype response with the Tc-99m MAb is novel since our MAb has not been fragmented and should theoretically produce the same pattern of HAMA.

Table 3: HAMA status and Survival times of ovarian cancer patients injected with OC-125 and B43.13

Patent #	Stage of Cancer	Number of MAb Injections[A]	HAMA Idiotype Positive	Survival Time (Months)[B]
1	IV	2	YES	43+
2*	III	2	YES	27+
3	I/II	2	YES	41+
4*	I/II	5	YES	27+
5	I/II	2	YES	44+
6	III	2	YES	45+
7	IV	2	YES	14
8	I/II	2	YES	52+
9*	III	2	YES	16+
10*	III	2	YES	35+

A All patients were injected with 1 mg of MAb OC 125 F(ab')2 per dose. Patients marked with a * also received 2 mg of MAb B43, reduced, unfragmented antibody.

B Patients are listed with a + if they are ongoing in the study. Patients listed without a + are deceased.

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CLAIMS

1. Use of an antibody in at least partially reduced form in the manufacture of a composition for the treatment of a disease associated with an antigen specifically bound by said antibody, said reduced antibody eliciting at least some anti-idiotypic anti-antibody response in a subject having said disease, but no more than a substantially decreased, if any, anti-isotype anti-antibody response, relative to the response which said antibody would have elicited had it been adaministered without said reduction.

2. The use of claim 1 wherein the antibody is repeatedly administered to the subject.

3. The use of claim 1 wherein the disease is a cancer and the antibody recognizes a tumor-associated antigen.

4. The use of claim 3 in which the cancer is an ovarian cancer.

5. The use of claim 3 in which the cancer is an adenocarcinoma.

6. The use of claim 1 in which the antibody is reduced with an agent selected from the group consisting of formamidine sulfonic acid, mercurous ion, stannous ion, cyanide ion, sodium cyanoborohydride, sodium borohydride, dithiothreitol, mercaptoethanol, mercaptoethanolamine, and thioredoxin.

7. The use of claim 1 in which the antibody is reduced with stannous ion.

8. The use of claim 6 in which the reduced antibody is labeled with technetium or rhenium.

9. A method of partially reducing an antibody which comprises reacting the antibody with a source of stannous ion in a tartrate buffer containing greater than 0.05M tartrate.

10. A method of radiolabeling an antibody which comprises partially reducing the antibody by the method of claim 9 to obtain an antibody with at least one free sulfhydryl group, and then reacting the partially reduced antibody with a pertechnetate or perrhenate salt to obtain a technetium- or rhenium-labeled antibody.

11. Use of a radiolabeled antibody in at least partially reduced form in the manufacture of a composition for the immunodetection by <u>in vivo</u> imaging of a disease associated with an antigen specifically bound by said antibody, said reduced antibody eliciting at least some anti-idiotypic anti-antibody response in a subject having said disease, but no more than a substantially decreased, if any, anti-isotype anti-antibody response, relative to the response which said antibody would have elicited had it been administered without said reduction.

ANHANG

zum internationalen Recherchenbericht über die internationale Patentanmeldung Nr.

ANNEX

to the International Search Report to the International Patent Application No.

ANNEXE

au rapport de recherche international relatif à la demande de brevet international n°

PCT/CA 93/00110 SAE 72361

In diesem Anhang sind die Mitglieder der Patentfamilien der im obengenannten internationalen Recherchenbericht angeführten Patentdokumente angegeben. Diese Angaben dienen nur zur Unterrichtung und erfolgen ohne Gewähr.

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The Office is in no way liable for these particulars which are given merely for the purpose of information.

La présente annexe indique les membres de la famille de brevets relatifs aux documents de brevets cités dans le rapport de recherche international visée ci-dessus. Les reseignements fournis sont donnés à titre indicatif et n'engagent pas la responsibilité de l'Office.

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
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